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ICAM-1 Participates in the Entry of West Nile Virus into the Central Nervous System▽

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Determining how West Nile virus crosses the blood-brain barrier is critical to understanding the pathogenesis of encephalitis. Here, we show that ICAM-1$^{-/-}$ mice are more resistant than control animals to lethal West Nile encephalitis. ICAM-1$^{-/-}$ mice have a lower viral load, reduced leukocyte infiltration, and diminished neuronal damage in the brain compared to control animals. This is associated with decreased blood-brain barrier leakage after viral infection. These data suggest that ICAM-1 plays an important role in West Nile virus neuroinvasion and that targeting ICAM-1 signaling may help control viral encephalitis.

West Nile virus (WNV) is the major cause of mosquito-borne encephalitis in North America (22). WNV enters the central nervous system (CNS) and may use several strategies to cross the blood-brain barrier (BBB). It is hypothesized that WNV may directly infect microvascular endothelial cells, cross endothelial cell junctions, or migrate within infected leukocytes that enter the CNS (6, 20, 21, 27). The leukocytic infiltration may also aggravate CNS inflammation, thereby contributing to encephalitis (20, 27). Viral entry by retrograde axonal transport via the peripheral nervous system may also occur (28).

Adhesion molecules on vascular endothelial cells and leukocytes play important roles in leukocyte traffic into the brain. Intercellular adhesion molecule 1 (ICAM-1, or CD54) is one of the most important molecules in this process (7, 8, 15). Up-regulation of ICAM-1 on CNS vascular endothelium and leukocyte-mediated breakdown of the BBB followed by leukocyte recruitment into the CNS are characteristics of brain inflammation (8, 24). Blocking ICAM-1 significantly inhibits the binding of lymphocytes to endothelial cells, as well as migration across the activated monolayer (8, 25, 30). ICAM-1 is also the receptor for human rhinovirus (2) and can incorporate into human immunodeficiency virus type 1 virions to enhance viral infectivity (16, 26). In addition, ICAM-1 knockout mice have decreased susceptibility to high doses of bacterial infection due to reduced T-cell activation and neutrophilic infiltration (33). These findings suggest that blocking ICAM-1 signaling may benefit the host, either by altering pathogen transmission or by controlling excessive immune responses. In this study, we examined the role of ICAM-1 in the pathogenesis of murine WNV encephalitis.

ICAM-1$^{-/-}$ mice are more resistant to lethal WNV infection. To determine whether WNV influences ICAM-1 expression, a murine brain endothelial cell line (bEnd.3; ATCC) was incubated with WNV isolate 2741 (multiplicity of infection, 0.1) for 24 h, and C57BL/6 mice were challenged intraperitoneally with 2×10^3 PFU of virus. The expression of ICAM-1 in the endothelial cell line or brain tissue was examined by quantitative PCR (Q-PCR). ICAM-1 was up-regulated both in vitro (Fig. 1a) and in vivo (Fig. 1b) after WNV infection, suggesting that it may be important in the course of disease. To examine the role of ICAM-1 in pathogenesis, ICAM-1$^{-/-}$ mice (no. 002867; Jackson Laboratory) and control animals were challenged with virus. Groups of 5 to 10 mice were inoculated with 2×10^3 PFU of WNV and monitored daily for mortality. As shown in Fig. 1c, ICAM-1$^{-/-}$ mice had an increased survival rate compared with control animals ($P < 0.01$).

ICAM-1$^{-/-}$ mice have a reduced viral load and immune pathology in the brain. To determine the reason for the resistance of ICAM-1$^{-/-}$ mice to WNV, the viral load and selected inflammatory/antiviral cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-6, gamma interferon (IFN-γ), and IFN-β1, were examined peripherally and in the CNS. The viral burden was measured by Q-PCR using the WNV envelope (WNVE) gene and normalized with the murine β-actin gene. The cytokine levels were measured by enzyme-linked immunosorbent assay or Q-PCR as described previously (32). The peripheral viral load and cytokine levels were similar in wild-type and ICAM-1$^{-/-}$ mice (Fig. 2). In contrast, the virus burden (Fig. 3a and b) and TNF-α, interleukin-6, and IFN-β1 levels (Fig. 3c and d) in brains from ICAM-1$^{-/-}$ mice were significantly lower than in those from wild-type animals.

To further characterize CNS infection, immunostaining of WNVE, CD45 (leukocyte common antigen), and CD11b (a macrophage marker) in frozen sections of murine brains was performed. Specimens were probed with anti-CD45 (clone YW 62.3; Serotec), anti-CD11b (clone M1/70.15; Serotec), and biotinylated WNVE antibody (32). After reaction with the proper fluorophore-labeled secondary antibodies, sections were examined with an Olympus BX-61 microscope. As shown in Fig. 3e and f, leukocyte and macrophage infiltrates were decreased in the brains of ICAM-1$^{-/-}$ mice compared with wild-type samples, and this was most evident in the olfactory bulb. The results also revealed a subset of CD45-positive leu-

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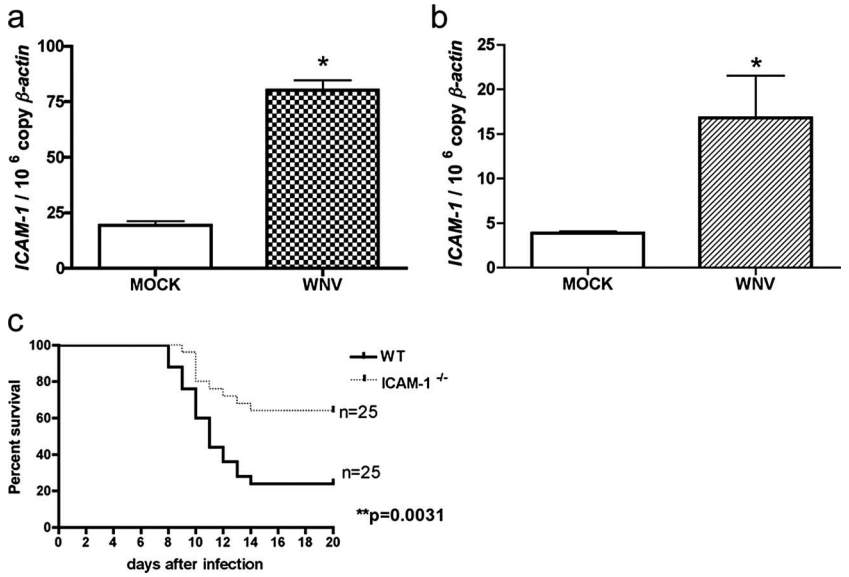

FIG. 1. ICAM-1 $^{-/-}$ mice are resistant to lethal infection with WNV. (a and b) ICAM-1 expression in a murine brain endothelial cell line after WNV stimulation (a) and in murine brain tissue at day 7 following virus infection (b) (*, $P < 0.05$; t test). The error bars indicate standard deviations. (c) The course of WNV infection in ICAM-1$^{-/-}$ mice. Wild-type and ICAM-1$^{-/-}$ mice were infected with 2×10^3 PFU of virus and monitored daily for mortality. The data shown are pooled from three independent experiments (**, $P < 0.01$; log rank test).

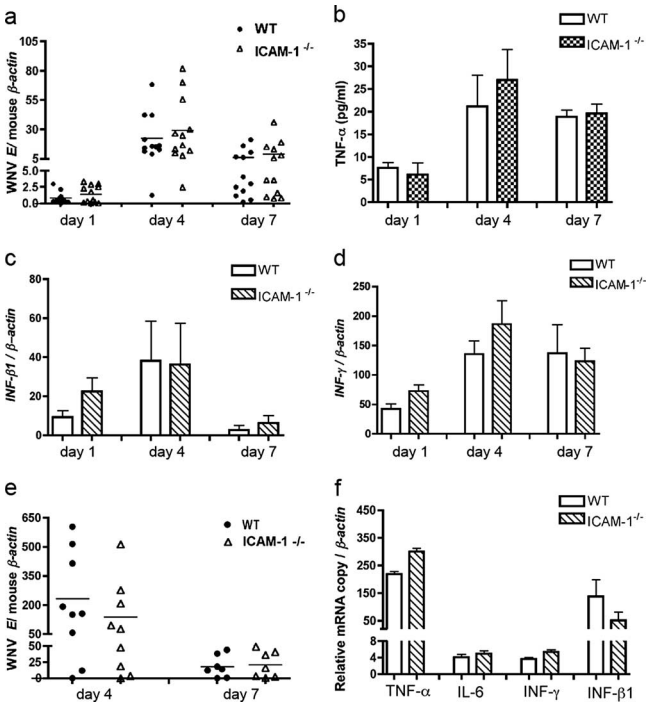

FIG. 2. ICAM-1$^{-/-}$ and wild-type (WT) mice have similar peripheral viral loads and cytokine levels after WNV infection. (a) WNV burdens in blood at days 1, 4, and 7 postinfection. The viral loads were determined by Q-PCR measuring the WNVE gene copies and normalized using the murine β-actin gene. Horizontal lines represent the mean values of the spots. (b) TNF-α levels in blood, measured by enzyme-linked immunosorbent assay. The error bars indicate standard deviations. (c and d) IFN-β1 and IFN-γ levels in blood, measured by Q-PCR. (e) WNV burdens in spleens at day 4 and day 7 postinfection. (f) Cytokine levels in spleens at day 7 postinfection. $P > 0.05$; t test (this P value is valid for all the data in the figure).

kocytes (especially CD11b-positive macrophages) that stained positively for WNV, suggesting an indirect mechanism whereby infected peripheral leukocytes can act as carriers of virus to the brain (9). Hematoxylin and eosin staining also showed that wild-type mice have more cellular infiltration and neuronal damage in the cerebral cortex after WNV infection than ICAM-1$^{-/-}$ mice (Fig. 3g).

ICAM-1 signaling contributes to WNV entry into the brain by enhancing permeability of the BBB. Since ICAM-1$^{-/-}$ and control mice had similar levels of virus in the blood and peripheral tissues but the ICAM-1$^{-/-}$ mice had less virus in the CNS, we hypothesized that BBB permeabilities are different in wild-type and ICAM-1$^{-/-}$ mice after WNV infection. We administered Evans blue, a dye normally excluded from the CNS, into WNV-infected wild-type and ICAM-1$^{-/-}$ mice through an intraperitoneal injection at day 3, when the viral load is high in the periphery, and at day 5, when the virus is detected in the brain (13, 18). An increased amount of Evans blue dye was found in brains from wild-type compared with ICAM-1$^{-/-}$ mice (Fig. 4a). In addition, the immunoglobulin G (IgG) level, another marker for BBB leakage, was much higher in brains from wild-type mice than in those from ICAM-1$^{-/-}$ mice (Fig. 4b).

Previous work showed that WNV infection can rapidly upregulate the expression of adhesion molecules, but the functions of these molecules in WNV pathogenesis remained largely unknown (20, 29). In this study, we showed that ICAM-1 plays an important role in WNV neuroinvasion. ICAM-1 is crucial for leukocyte infiltration into the brain, and the process of leukocyte migration across the brain endothelial layer can accelerate the BBB breakdown (3, 8). Recent studies alsosuggest that ICAM-1 not only acts as a ligand for leukocyte receptors, but also can serve as a signal transducer that influences BBB permeability and the progression of neuroinflammation (10, 15, 17). Blocking ICAM-1

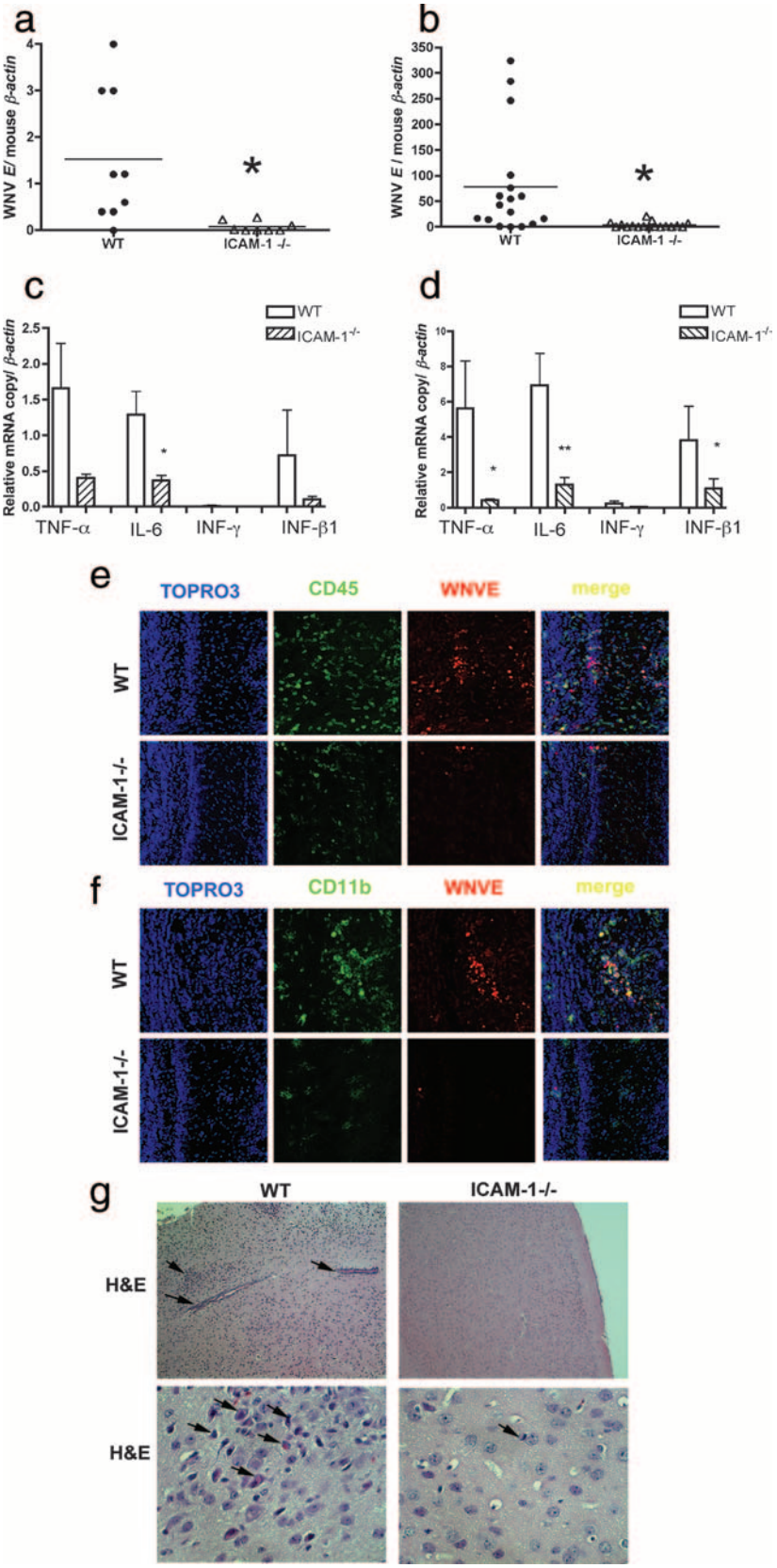

FIG. 3. Reduced viral loads and neuronal pathology in brains of WNV-infected ICAM-$1^{-/-}$ mice. (a and b) WNV burdens in brains at days 5 (a) and 7 (b) postinfection. (c and d) Cytokine levels in brains at day 5 (c) and day 7 (d) postinfection (*, $P < 0.05$; **, $P < 0.01$; *t* test). WT, wild type. (e and f) Immunofluorescence micrographs of olfactory bulbs at day 7 postinfection double stained for WNVE antigen (e and f) and either CD45 (e) or CD11b (f). Nuclei were stained with TOPRO3 (magnification, ×250). (g) Hematoxylin and eosin staining in cerebral cortices at day 7 postinfection. The arrows indicate the sites of perivascular mononuclear infiltration (top) (magnification, ×50) and damaged neurons, characterized by shrunken and heavily stained somata (bottom) (magnification, ×400).

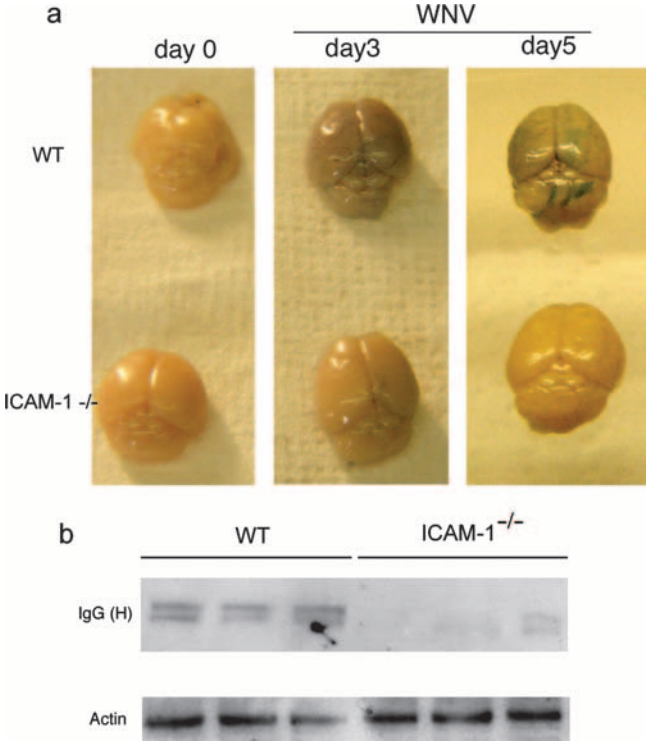

FIG. 4. Reduced BBB leakage in ICAM-$1^{-/-}$ mice after WNV infection. (a) Evans blue staining of WNV-infected whole brains (days 0, 3, and 5). WT, wild type. (b) Total IgG in brains at day 7 postinfection, measured by Western blotting (showing IgG heavy chain [H]). The data shown are representative of at least three independent experiments.

can reduce the relative numbers of infiltrating leukocytes, as well as attenuating the BBB leakage caused by its downstream signaling. Our experiments showed that ICAM-$1^{-/-}$ mice had a more intact BBB than wild-type mice after WNV infection. Our data suggest that this results in reduced virion influx across a compromised BBB, decreasing leukocyte infiltration and virus entry into the brain carried by infected immune cells and attenuating the pathological effect caused by immune cell infiltration. The outcome of WNV infection is clearly a balance between eradication of virus and virus- or immune-mediated tissue damage (14, 20, 27). A similar study was done in β2-integrin- and ICAM-1-deficient mice using lymphocytic choriomeningitis virus infection. The results also showed decreased mortality in the knockout mice after infection, and at the same time, the T-cell response was almost intact (5).

Targeting ICAM-1 has been shown to be promising in controlling multiple inflammatory and infectious diseases. For example, ICAM-1 neutralizing antibody blocks cell-associated human immunodeficiency virus type 1 transmission across a cervical epithelial monolayer (4). Modulating ICAM-1 signaling may also alleviate the outcomes of many diseases, such as colonic inflammation, asthma, and polymicrobial sepsis (1, 11, 12, 19, 23, 31). Our current findings support an important role of ICAM-1 in WNV neuroinvasion and also suggest the potential application of modulating ICAM-1 signaling in controlling the pathogenesis of West Nile encephalitis and other neuroinvasive flaviviral infections.

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